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Housekeeping gene variability in normal and cancerous colorectal, pancreatic, esophageal, gastric and hepatic tissues

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Abstract

Careful normalization is essential for the accurate quantitation of mRNA levels in biopsy-sized tissue samples. Commonly, normalization of the target gene with an endogenous standard, mainly housekeeping genes (HKGs), is applied. However, differences in the expression levels of endogenous reference genes have been reported between different tissues and pathological states. Therefore, we were challenged to identify a set of endogenous reference genes whose mRNA expression levels would not change significantly between normal and cancerous tissues. Quantitative real-time PCR (Q-RT-PCR) analysis was applied to evaluate the variability in gene expression among 21 classical housekeeping genes in colorectal, pancreatic, esophageal and gastric cancer as well as in liver metastases in comparison to the corresponding normal tissue. Our results indicated that some housekeeping genes were candidates with relatively stable gene expression in several of the investigated tissues but for most of the HKGs under investigation our data have revealed distinct differences in the extent of variability in gene expression between the different tissues and pathological states. However, for each of the five tissues investigated we found a group of genes that were expressed at a constant level thus representing a panel of candidates that we can recommend as housekeeping genes in the respective tissue types. In summary, our results can be used as guidance for other scientists studying various carcinomas for tissue-specific selection of the optimal housekeeping gene (HKG) to be used in normalizing target gene expression.

Keywords: Quantitative real-time PCR; mRNA quantification; Cancer biopsies; Reference genes; Gene expression analysis; Normalization

1. Introduction

Several methods have been developed to study the regulation of gene expression and to identify and quantitate mRNA species that code for specific proteins. Among these methods, at present, the most common method for the quantification of individual genetic differences in tumor versus uninvolved tissue and the examination of gene expression patterns in different sample populations is the fluorescence-based quantitative real-time PCR (Q-RT-PCR) [1]. Generally two quantification types in Q-RT-PCR are possible: absolute quantification, which determines tile input copy number, usually by relating the PCR signal to a standard curve and relative quantification. which relates the PCR signal of the target transcript in a treatment group to that of another sample such as an untreated control [2]. For relative quantification the accurate determination of gene expression levels requires tile use of an ubiquitously expressed internal control, such as mRNA specific for a housekeeping gene (HKG), ribosomal RNA (rRNA) or accurately quantitated total RNA in order to normalize the amount of total RNA in unknown samples.

Using Q-RT-PCR assays to compare mRNA levels between biopsies from different individuals and disease states, careful normalization to an endogenous reference is essential for correcting results of differing amounts of input RNA, uneven loading between samples and variation of

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experimental conditions [3]. Ideally, internal standards should be constitutively expressed by all cell types independent of experimental conditions and they should not be affected by any human disease. Some of these criteria are presumptively fulfilled by a number of housekeeping genes (HKGs) commonly used as endogenous references and present in all nucleated cell types since they are necessary for basic cell survival. The mRNA synthesis of these genes is putatively considered to be stable and secure in various tissues, even under experimental treatments. However, in practice this is often not the case and the choice of an appropriate reference gene is still the subject of debate as expression level variations may limit the usefulness of some currently used HKGs. Numerous studies have already shown that many of the commonly used HKGs are regulated and vary under experimental conditions [4–6]. This may partly be explained by the fact that housekeeping proteins are not only implicated in the basal cell metabolism but also participate in other cell functions [7]. Even widely used reference genes like β-actin (ACTB) [8–10,14], cyclophilin (CYCC) [10,14], tubulins [11], glyceraldehyde-3-phosphate (GAPDH) or hypoxanthine phosphoribosyltransferase (HPRT) were criticized in the past because of varying expression levels under different experimental conditions and diseased states [12–14]. GAPDH for instance as a key enzyme in glycolysis constitutively expressed in many tissues is still widely used as an internal standard even though wide variations in GAPDH expression levels have been observed in tumor cell lines [15], tissues at different developmental stages [16], under hypoxia [13] and in human lung cancer tissues arid liver metastases compared with normal lung and liver tissues, respectively [9,13,17].

In order to evaluate which reference genes may be suitable candidates for normalization we examined the variability of gene expression in tissue samples of five different tissue types. Each tissue type represented 10 patient cases. Total RNA was isolated from tissue samples including primary colorectal cancer, colorectal liver metastases as well as pancreatic, esophageal and gastric cancer, plus the corresponding normal tissue samples. Q-RT-PCR analysis was then used to evaluate the variability in gene expression among the HKGs under investigation in order to determine the extent of variability in gene expression between normal and malignant tissue.

Before starting a relative quantification approach with a target gene and a HKG as a reference, it is essential that the amplification efficiency of both genes is tested. Only if the slopes of the standard curves of both genes differ less than 0.1, the respective HKG may be used in the experiment [2]. Hence, a panel of HKGs is needed to choose a suitable candidate from because not only the lowest variability in gene expression is demanded for a HKG to be suitable for normalization but also compatible amplification efficiencies are very important to produce correct results. Testing a panel of HKGs each time before starting a Q-RT-PCR experiment with a target gene is very time-consuming and cost-intensive and therefore we decided to do a survey of a broad range of HKGs according to their suitability in various tissues. With their amplification efficiencies being tested and known we are now provided with a wide selection of HKGs to quickly choose a suitable candidate from for the various target genes and tissues we are interested in. All the HKGs used in this study were selected according to their biological functions and roles in biochemical pathways in order to obtain a wide range of putative candidates for profiling the expression of target genes, thus, providing other scientists with a solid guideline to the selection of appropriate HKGs. The HKGs we chose to investigate are presented in Table 1, listing their biological functions and the processes in which they are involved. Hence, we found a number of HKGs for each tissue type examined with very little variation in gene expression between malignant and corresponding normal tissue. However, our data also show that some of these HKGs are suitable candidates in some tissues showing only little variability between normal and malignant tissue whereas the same HKGs may show clear differences in gene expression in other tissue types irrespective of their biological function. Consequently, not one single HKG always manifests stable expression levels in all tissue types under investigation, therefore, emphasizing the necessity to characterize the suitability of various HKGs to serve as internal controls in the respective tissue type where transcription effects are tested.

2. Materials and methods

2.1. Tissue preparation

Following an institutionally approved protocol, informed consent was obtained from cancer patients undergoing surgical resection at our department from December 2001 to July 2003. Tissues from primary esophageal, pancreatic, gastric and colorectal carcinoma as well as colorectal liver metastases were aquired. In order to keep warm ischemia time short (usually below 2 min), all samples were handed over for further processing right from the surgeon removing the tumor. As corresponding normal tissue we used healthy tissue from the same resection specimen. All tissues obtained were reviewed by a surgical pathologist and examined for the presence of tumor cells. As a minimum criteria for usefulness for our studies we only chose tumor tissues in which tumor cells occupied a major component of the tumor biopsy. Immediately after resection, tissue samples were frozen in liquid nitrogen and then stored at −80 °C until RNA was being extracted.

2.2. Isolation of total RNA

Total RNA was isolated using RNeasy columns from Qiagen (Hilden, Germany) following the manufacturer's

Table 1
Housekeeping genes examined

Symbol	Name	Accession number	Function	Process
18S rRNA	18S ribosomal RNA	X03205	Part of a ribosomal subunit	Translation
QRRS	Glutaminyl-tRNA synthetase	NM_005051	Catalyzes the aminoacylation of tRNA (Gln and Glu)	Translation
PGK	Phopsphoglycerokinase	NM_000291	Key enzyme involved in glycolysis following GAPDH, carbohydrate kinase	Glycolysis
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046	Dehydrogenase, oxidoreductase in glycolysis and gluconeogenesis	Glycolysis
HPRT	Hypoxanthine phosphoribosyl-transferase I	NM_000194	Glycosyltransferase, purine synthesis in salvage pathway	Nucleotide-metabolism
ADA	Adenosine deaminase	NM_000022	Deaminase, purine metabolism, catalyzes the hydrolysis of adenosine to inosine	Nucleotide-metabolism
TAF2	TAF2 RNA polymerase II, TATA box binding protein (TBP)-associated factor	NM_003184	DNA-directed RNA polymerase, cofactor of the RNA polymerase II transcription factor	Transcription
POLR2L	Polymerase (RNA)II polypeptide L	NM_021128	Subunit of RNA polymerase II	Transcription
CETN2	Caltractin, centrin	NM_004344	Structural component of the centrosome, calmodulin related protein	Cytoskeleton, cell cycle, chromosome segregation
ACTB	β-Actin	NM_001101	Cytoskeletal protein, essential for the structure and kinetics of the cytoskeleton	Cytoskeleton, cell structure, cell locomotion
UBE2D2	Ubiquitin-conjugating enzyme E2D 2	NM_003339	Member of the ubiquitin-conjugating enzyme E2 subfamily, may catalize ubiquitination of cellular proteins prior to degradation	Proteolysis
PSMB6	Proteasome subunit Y	NM_002798	Protein degradation, proteasome	Proteolysis
CAPN2	Ca-activated neutral protease large subunit (calpain 2)	NM_001748	Calmodulin related protein, Ca-activated neutral non-lysosomal intracellular cysteine-type proteases	Protcolysis
TXNRD1	Thioredoxin reductase 1	NM_003330	Reductase, involved in maintaining redox balance, oxidoreductase family	Sulfur redox metabolism
SDHA	Succinate dehydrogenase complex, subunit A	NM_004168	Dehydrogenase, electron transporter in the tricarboxylic acid pathway and respiratory chain	Oxidative phosphorylation, electron transport
GUS	β-Glucuronidase	NM_000181	Glycoprotein, exoglycosidase in lysosomes	Glycosylation
CYCC	Cyclophilin C, (peptidylprolyl-isomerase C)	NM_000943	Isomerase, involved in diverse cellular protein interactions	Protein folding, nuclear transport, T cell mediated immunity
PMM1	Phosphomannomutase I	NM_002676	Mutase, mannose-1-P phosphorylation	Monosaccharide metabolism
AGPATI	Lysophosphatidic acid acyltransferase	NM_006411	Acyltransferase, converts LPA into PA, both involved in signal transduction and lipid biosynthesis	Phospholipid metabolism
HDAC10	Histone deacetylase	NM_0320l9	Deacetylase, removes acetate from histone, which is important for regulating chromatin structure and gene expression, (interaction with retinoblastoma tumor suppressor protein!)	Histone deacetylation
β2M	β2-Microglohulin	NM_004048	Major histocompatibility complex antigen, involved with immune response, major component of the hemodialysis-associated amyloid fibrils	MHC-mediated immunity

instructions and all samples were treated with RNase-free DNase to prevent amplification of genomic DNA that may be contained in samples. Samples were dissolved in RNase-free water and quantified by the average of triplicate spectophotometric readings at 260 nm (A_{260}). Purity of total RNA was determined by the A_{260}/A_{280} and A_{260}/A_{230} ratio, respectively. Before cDNA synthesis, the integrity of RNA samples was confirmed by electrophoresis on 1% agarose gels.

2.3. Single-strand cDNA synthesis

All RT-PCR reagents were purchased from Applied Biosystems (Foster City, CA). For cDNA synthesis 5 μg of each patient total RNA sample were reverse-transcribed in a final reaction volume of 50 μ1 giving a final concentration of 100 ng/μl thus insuring the equalization of quantities of input RNA. The reaction assay contained 1×TaqMan RT buffer, 2.5 μM random hexamers, 500 μM each dNTP,

5.5 mM $MgC1_2$, 0.4 U/μlRNase inhibitor, and 1.25 U/μl Multiscribe RT and the reaction conditions were 10 mm at 25 °C, 30 mm at 48 °C, and 5 min at 95 °C.

2.4. Principle of real-time PCR (TaqMan)

The theoretical basis of the ABI PRISM 7900 Sequence Detection System (TaqMan) real-time quantitative PCR (Applied Biosystems, Foster City, CA) is described in detail elsewhere [18]. Fluorescent signal from each PCR reaction is collected as peak-normalized values plotted versus the cycle number. Reactions are characterized by comparing threshold cycle (C_T) values. The C_T value is a unitless value defined as the fractional cycle number at which the target fluorescent signal passes a fixed threshold above baseline.

2.5. Primers and probe design

Except for human PGK (NM_000291) all other HKGs were purchased as 'assays on demand' (Applied Biosystems, Foster City, CA) and utilized according to the manufacturers instructions. The primers and probes for PGK were designed in our laboratory using the Primer Express software (Applied Biosystems, Foster City, CA), and synthesized and HPLC purified by MWG Biotech (Ebersberg, Germany). The probe had an amplicon size of 81 bp and was labeled with Joe at the 5′-end and BHQ1 (black hole quencher) at the 3′-end.

Forward primer: 5′-AGGAAGAAGGGAAGGGAA-AAGA-3′
Reverse primer: 5′-GTGAAGCTCGGAAAGCTTC-TATTT-3′
Probe: 5′ Joe-CTTCTGGGAACAAGGTTAAAGCC-GAGCC-3′ BHQ1

2.6. Quantitative PCR analysis

Prior to Q-PCR analysis, all patient cDNA samples except 18S ribosomal RNA (18SrRNA) were diluted 1:2 with RNase-free water to a final concentration of 50 ng/μl. 18SrRNA patient cDNA samples were diluted 1:10 with RNase-free water to yield a final concentration of 5 ng/μ1. Subsequently all PCR reactions except for PGK were carried out using 12.5 μ1 2× Taqman PCR Universal Master Mix No AmpErase® UNG (Applied Biosystems, Foster City, CA), 1.25 μl assay on demand containing the primers and probe mix (Applied Biosystems, Foster City, CA), 10.25 μ1 RNase-free water and 1 μ1 cDNA template. PCR reactions for PGK were carried out using 12.5 μ1 2× Taqman PCR Universal Master Mix No AmpErase® UNG (Applied Biosystems, Foster City, CA), 2.25 μ1 Forward Primer (10 μM, MWG), 2.25 μl Reverse Primer (10 μM, MWG), 0.63 μl probe (10 μM, MWG), 6.37 μl RNase-free water and 1 μl cDNA template. Amplification was performed in a ABI Prism 7900 sequence detection system in 96-well microtitre plates using aliquots of 25 μl each corresponding to 50 ng RNA of the initial sample except for I 8SrRNA, for which a final concentration of 5 ng template was applied. All reactions were run in triplicates along with no template controls on each microtiter plate and an additional reaction in which reverse transcriptase was omitted to allow for assessment of genomic DNA contamination in each RNA sample. For the signal detection, ABI Prism 7900 sequence detector was programmed to an initial step of 10 mm at 95 °C, followed by 40 thermal cycles of 15 s 95 °C and 10 min at 60 °C and the log-linear phase of amplification was monitored to obtain C_T values for each RNA sample.

2.7. Microdissection

Laser microbeam microdissection (LMM) was employed for obtaining pure tumor cell samples for subsequent genetic analysis. Immunohistochemical staining was used on membrane mounted paraffine tissue sections before microdissection. Specimen preparation, microdissection and catapulting were performed following a laser pressure catapulting protocol according to the manufacturers instructions (P.A.L.M. Microlaser Technologies, Bernried, Germany). RNA was extracted using the P.A.L.M. RNA extraction kit and for reverse transcription the invitrogen reverse transcription kit (Invitrogen Life Technologies, Karlsruhe, Germany) was applied. Subsequently quantitative PCR analysis was performed as described earlier.

2.8. Calculations and statistical methods

The comparative C_T method was employed to determine the variation between normal and malignant tissues among the 21 HKGs. Since each patient RNA sample (based on spectrophotometric analysis) contained the same starting amount of RNA, the variation between cancerous tissues relative to their corresponding normal tissues reflects the variation among HKG expression. Since reporting of data obtained from raw C_T values falsely represent the variations, we converted the individual C_T values to the linear form as follows:

$$\text{Fold difference} = 2 - (\text{mean } C_T \text{ pathological tissue} - \text{mean } C_T \text{ calibrator}) = 2 - \Delta C_T$$

Hence, the normal tissue became the 1× sample, and all other quantities were expressed as an n-fold difference relative to the normal tissue. The standard errors for each HKG were calculated based on Q-RT-PCR analysis of tissue samples for each of the normal and cancerous tissues ($n = 10$). Fold increase above 1 indicated HKG overexpression and fold decrease under 1 indicated HKG downregulation (Fig. 1).

3. Results

3.1. Quantitation of HKG expression in human tissues using Q-RT-PCR

Quantitative real-time PCR was used to measure the variability in RNA transcription levels of 21 HKGs in five different human tissues. Prior to variability studies the PCR efficiency of the assays was determined to ensure comparability between the 21 PCR assays. The average values of the expression levels for each HKG and the standard errors are shown for colorectal liver metastases (Fig. 1A) and malignant tissues of colon (Fig. 1B), stomach (Fig. 1C), pancreas (Fig. 1D) and esophagus (Fig. 1E). In some

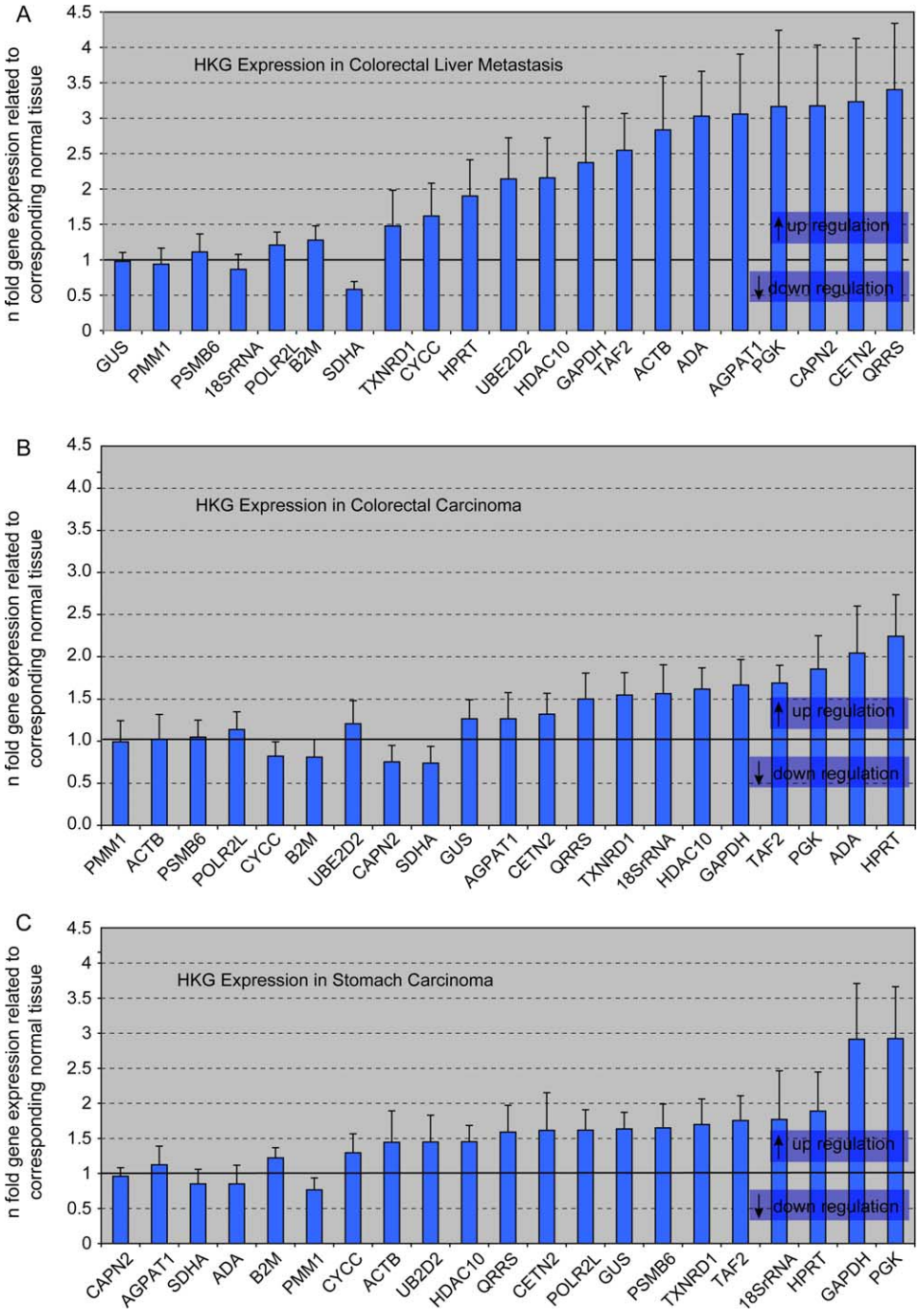

Fig. 1. Comparative expression of 21 HKGs as determined by Q-RT-PCR. (A) Normal and metastatic liver tissues. (B) Normal and colorectal cancerous tissues. (C) Normal and stomach cancerous tissues. (D) Normal and pancreatic cancerous tissues. (E) Normal and esophageal cancerous tissues. The standard errors for each HKG were calculated based on QRT-PCR analysis of tissue samples for each of the normal and malignant tissues ($n = 10$). Fold increase above 1 indicate HKG overexpression and fold decrease under 1 indicate HKG downregulation.

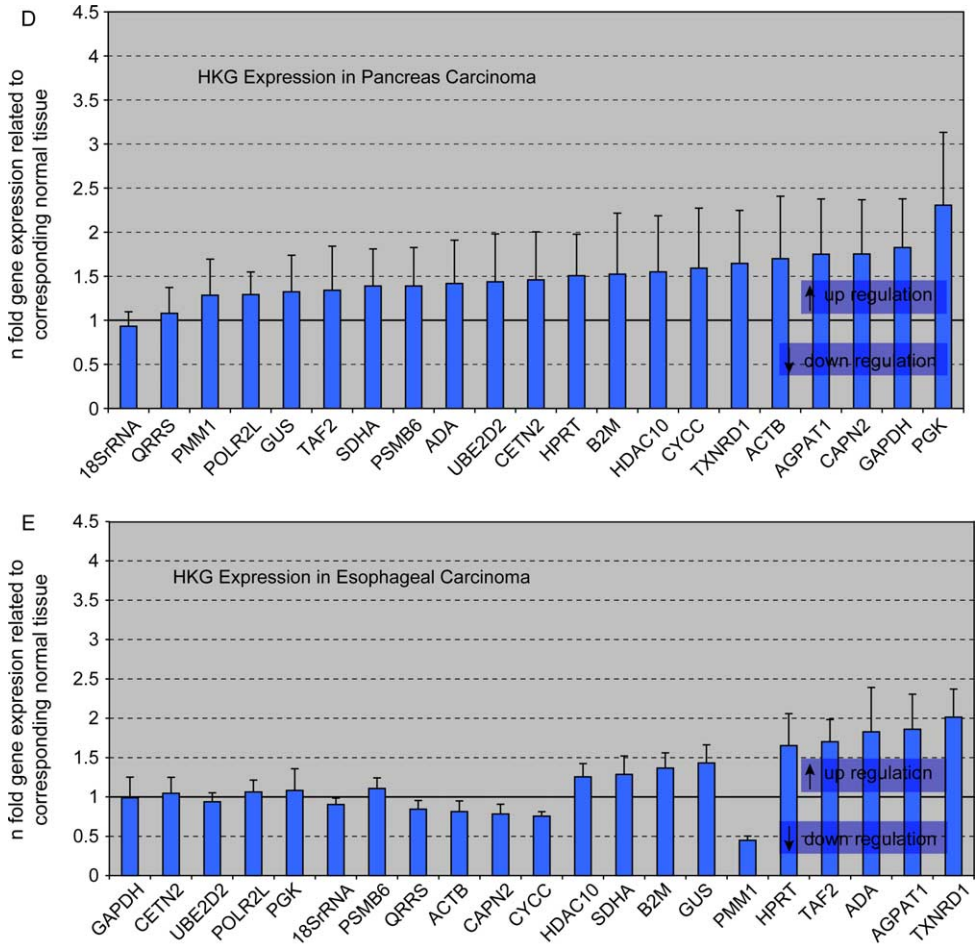

Fig. 1 (*continued*)

samples the tumor component was microdissected and the expression patterns in the tumor cells of all HKGs under investigation were compared. The obtained data corresponded well with the results presented in Fig. 1. The standard errors shown here represent the differences in HKG expression between different patient samples. Since we were aware of the problem that averaging out the C_T values or ratios could mask significant differences between individual paired samples, we also analysed the differences between HKG expression from matched normal/cancer samples. The results obtained from this level of analysis corresponded widely with the results presented in Fig. 1 thus ensuring that the expression profile holds true. As degradation of the RNA directly affects A_{260} measurements we assessed the level of degradation by electrophoretic investigations. The results confirmed only minimal degradation throughout our samples. Since there would be a greater chance for the tumor tissue to resemble the normal tissue in terms of most expressed genes if tumor cells only occupied a minor component of the tumor biopsy, we only chose tumor tissues in which tumor cells occupied on average 90% of the tumor biopsy.

Among the 21 HKGs under investigation (Table 1), Glutaminyl-tRNA Synthetase (QRRS), centrin (CETN2), calpain 2 (CAPN2) and Phosphoglycerokinase (PGK) showed the highest variability in expression in the colorectal liver metastases compared to normal liver tissue, demonstrating 3.4-, 3.3- and 3.2-fold expression increases, respectively (Fig. 1A). In contrast, β-Glucuronidase (GUS), phosphomannomutase 1 (PMM 1), proteasome subunit Y (PSMB6) and 18SrRNA expression remained relatively constant (less than 2-fold variation) (Fig. 1A). In malignant colon samples PMM1, ACTB and PSMB6 showed variations of less than 10% and therefore, the lowest variability in expression followed by a wide range of genes demonstrating intermediate variability in expression ranging from 1.2- – 2-fold between cancerous and normal colon tissue expression (Fig. 1B). Showing more than 2-fold variation adenosine deaminase (ADA) and HPRT were the candidates with the highest variability in expression in colorectal carcinoma. Our analysts in gastric carcinoma tissues revealed that all HKGs evaluated demonstrated variations of less than 2-fold except GAPDH and PGK which showed 3-fold expression increases (Fig. 1C). Studies in pancreatic carcinoma tissues demonstrated for all HKGs under investigation variations of less than 2-fold except for PGK which showed more than 2-fold expression increases (Fig. 1D). Similar to our results in gastric

carcinoma the second highest variation of 1.8-fold was displayed by GAPDH which therefore shows together with PGK the highest variability in expression in pancreas carcinoma tissues. 18SrRNA and QRRS demonstrated variations of less than 10% and therefore, the lowest variability in expression between cancerous and normal pancreas tissue. In esophageal carcinoma tissues the expression of the majority of HKGs under investigation remained relatively constant (less than 1.5-fold variation) with GAPDH, CETN2, Ubiquitin-conjugating enzyme E2D2 (UBE2D2). Polymerase (RNA)II polypeptide L (POLR2L), PGK and 18SrRNA displaying less than 10% variability in expression between cancerous and normal esophageal tissue (Fig. 1E). It is notable that GAPDH and PGK were among the genes with the lowest variability in expression in the esophageal tissues since in all other tissues tested these two genes were among the candidates with the highest variability of expression. Showing variation within a range of approximately 1.5–2-fold PMM1, HPRT, TAF2 RNA polymerase II TBP-associated factor (TAF2), ADA, Lysophosphatidic acid acyltransferase (AGPAT1) and thioredoxin reductase 1 (TXNRD1) demonstrated the highest variability in expression between malignant and normal esophageal tissue.

4. Discussion and conclusions

An ideal housekeeping gene should maintain a constant RNA transcription level in all cell types and tissues, it should be resistant to regulative factors and its expression should not vary due to treatment. Hence the purpose of this study was to evaluate the variation in expression among 21 different HKGs in normal and malignant tissues of pancreas, stomach, esophagus, primary colon cancer and colorectal liver metastases and to determine the optimal candidates for each tissue type.

The HKGs we investigated fall roughly into eight different groups: glycolysis-related genes: PGK and GAPDH; transcription/translation-related genes: 18SrRNA, QRRS, TAF2 and POLR2L; structure/cytoskeleton-related genes: CETN2, ACTB; genes involved in proteolysis: CAPN2, UBE2D2 and PSMB6; genes involved in the redox household: TXNRD1 and succinite dehydrogenase (SDHA); lysosomal enzymes: GUS; nucleotide-metabolism related genes: HPRT and ADA and finally the genes that do not clearly categorise into one of these groupings including β2M-microglobulin (β2M), AGPAT1, PMM1, histone deacetylase (HDAC10) and CYCC (Table 1).

The HKGs that code for the metabolic enzymes, PGK and GAPDH, demonstrated high up-regulation in cancerous versus normal tissues from pancreas, stomach, colon and colorectal liver metastases. However, in malignant esophageal tissues, PGK was only slightly overexpressed and GAPDH was the least variable of the 21 HKGs. Even though it is now well documented that GAPDH mRNA levels are not constant [9,15,19,20], it continues to be utilised as a normaliser despite continuing reports that emphasize the problems associated with its use. There are some instances when normalisation to GAPDH may be valid [21,22], but for most experimental conditions its use is inappropriate. GAPDH is pathologically implicated in apoptosis and neurodegenerative disease [23] and its mRNA levels are highly heterogeneous even in cellular subpopulations of the same pathological origin [24].

The translational HKGs, QRRS and 18SrRNA, both demonstrated in pancreas and esophageal carcinoma only minimal variability of expression and in colorectal and stomach carcinoma they both showed intermediate variability of expression between cancerous and normal tissue samples. However, in colorectal liver metastases QRRS was the most variable of the 21 HKGs under investigation while the expression of 18SrRNA was shown to be relatively stable. The ribosomal HKG I 8SrRNA is often used as a reference and has been described as a preferable control [7] which is expressed at relatively constant levels in liver metastases compared to normal liver [13,15] or in other cancer types [24] which may result from its lack of involvement in cellular metabolism [25]. However, other studies demonstrated I 8SrRNA to be regulated [26] and regard it as an unsuitable reference because its synthesis is independent from synthesis of mRNA [27]. Since there is a very large discrepancy in expression levels between mRNA and rRNA, it is necessary to use template dilutions of 18SrRNA in the Q-RT-PCR steps thus enhancing the risk of introducing errors into the experiment. Therefore, we would not recommend the use of 18SrRNA despite the relatively stable expression in normal and malignant tissues that we demonstrated in our survey.

The two transcriptional HKGs, TAF2 and POLR2L, showed different expression levels in the various tissues under investigation. While POLR2L showed relatively stable gene expression in all tissues tested, TAF2 demonstrated higher but still intermediate variability of expression in colon, gastric, pancreas and esophageal carcinomas. Only in colorectal liver metastases compared to normal liver, TAF2 appears to be highly overexpressed. The general transcription factor TFIID and its individual subunits (TAF(II)s) have been the focus of many studies, yet their functions in vivo are not well established. In addition to core promoter selectivity the derepression of promoters was shown to be a further function [28,29]. Since transcriptional activity changes with the transformation of a normal cell into a malignant cell, differences in expression between normal and cancerous tissues may be expected. Still, cofactors of TFIID seem to underly a higher degree of variability in gene expression than POLR2L, a gene encoding a subunit of human RNA polymerase [30] as our results indicate.

Structural HKGs involved in the cytoskeleton like CETN2 and ACTB demonstrated less variation than the metabolic HKGs in most tissues under investigation, only in

colorectal liver metastases ACTB and CETN2 were highly overexpressed. These results are in compliance with previous data from Blanquicett et al. [13], who demonstrated even 5-fold overexpression for ACTB in colorectal liver metastases compared to normal liver whereas ACTB expression in colon carcinoma was shown to be stable.

The HKGs involved in proteolysis, CAPN2, UBE2D2 and PSMB6, demonstrated stable gene expression in colorectal and esophageal carcinomas and intermediate variability of expression in stomach and pancreas carcinomas. However, in colorectal liver metastases CAPN2 and UBE2D2 showed high overexpression compared to normal liver while variability of expression of PSMB6 remained very low. CAPN2 is a ubiquitous intracellular cytoplasmic non-lysosomal cysteine endopeptidase. Many known substrates of the different CAPN2 isoenzymes have been implicated in the pathogenesis of various malignancies of human skin, and alterations in CAPN2 mRNA expression and protein content have been shown in malignant skin tumours [31]. Since CAPN2 may be of importance for the tumorigenesis and growth not only of human skin malignancies but also for other neoplastic diseases, it may not be a suitable candidate as a HKG even though its expression seemed relatively stable in colorectal and esophageal carcinomas.

Enzymes involved in the redox household, such as TXNRD1 and SDHA, showed intermediate variability of expression in all tissues tested except in esophageal carcinoma where TXNRD1 showed higher variability of expression than in the other tissues.

A candidate with relatively stable expression in all live tissues under investigation was the lysosomal enzyme GUS, a HKG involved in carbohydrate metabolism. These data are in compliance with the results of other authors [15]. GUS even demonstrated minimal variation of expression between liver metastasis and normal liver as was demonstrated also by Blanquicett et al. [13].

Enzymes involved in the nucleotide-metabolism, such as the deaminase ADA and the glycosyltransferase HPRT, were clearly overexpressed in cancerous versus normal samples in all tissues under investigation. Only in cancerous tissues of the stomach ADA expression was shown to be relatively stable in normal and malignant tissue. The high variability of expression that was demonstrated in most tissues by HPRT and ADA was also found in other diseases [32] and is supported by several findings that describe the involvement of both enzymes in various malignant tissues and malignancies. Activities of adenosine deaminase enzymes and down-regulation of HPRT were measured in various malignant diseases [33–35] suggesting that both enzymes might be regulated in neoplastic tissues.

Among the HKGs under investigation that did not clearly categorise into a special grouping were PMM1 and ß2M candidates with relatively stable and low gene expression in most tissues under investigation. AGPAT1, HDAC and CYCC were candidates with intermediate variation in expression between normal and malignant tissues in most tissues but colorectal liver metastases, where all three enzymes showed elevated gene expression levels.

Since variability in the expression of HKGs may lead to false results, it is essential to choose the right HKG when normalizing RNA concentrations in Q-RT-PCR analyses. Our results indicated that POLR2L, PMM1, GUS, B2M and PSMB6 were candidates with relatively stable gene expression in several of the investigated tissues even though they fall into different functional groups. However, for most of the HKGs under investigation our data demonstrated notable differences in the extent of variability in gene expression between the different tissues and pathological states. It is therefore evident that the identification of a valid reference for data normalization to achieve accurate, reproducible, and biologically relevant mRNA quantification is critical to the interpretation of experimental results and remains a very important task, especially when comparing gene expression profiles using in vivo biopsies from different individuals. In conclusion, our approach has led to the discovery of a panel of endogeneous reference genes that were expressed at a constant level in different tissues and diseased states. Moreover, our results offer a validated overview of a wide range of HKGs in five selected tissues that we can recommend as HKGs for normalizing target gene expression in five different carcinoma types.

References

[1] Bustin SA. Quantification of mRNA using real-tune reverse transcription PCR (RT-PCR): trends and Problems. J Mol Endocrinol 2002;29:23–39.

[2] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\mathrm{deltaCt}}$ method. Methods 2001;25:402–8.

[3] Pfaffl W. A new mathematical model for relative quantification in real-time RT-PCR. Nucl Acids Res 2001;29:2002–7.

[4] Wu Y, Rees JL. Variation in epidermal housekeeping gene expression in different pathological states. Acta Derm Venerol 2000;80:2–3.

[5] Tricarico C, Pinzani P, Bianchi S, Paglierani M, Distante V, Pazzagli M, et al. Quantitative real-tine reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. Anal Biochem 2002;309:293–300.

[6] Beillard E, Pallisgaard N, Van der Velden VM, Bi W, Dee R, Van der Schoot E, et al. Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using real-time quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR)—a Europe against cancer program. Leukemia 2003;17: 2474–86.

[7] Thellin O, Zorzi W, Lakaye B. Housekeeping genes as internal standards: use and limits. J Biotechnol 1999;75:291–5.

[8] Foss DL, Baarsch MJ, Murtaugh MP. Regulation of hypoxanthine phosphoribosyltransferase, glyceraldehydes 3 phosphate dehydrogenases and beta-actin mRNA expression in porcine immune cells and tissues. Anim Biotechnol 1998;9:67–78.

[9] Suzuki T, Higgins PJ, Crawford DR. Control selection for RNA quantitation. Biotechniques 2000;29:332–7.

[10] Chang TJ, Juan CC, Yin PH, Chi CW, Tsay HJ. Up-regulation of beta-actin, cyclophilin and GAPDH in ns1s1 rat hepatoma. Oncol Rep 1998;5:469–71.
[11] Marten NW, Burke EJ, Hayden JM, Strass DS. Effect of amino acid limitation on the expression of 19 genes in rat hepatoma cells. FASEB J 2000;8:538–44.
[12] Lupberger J, Kreuzer KA, Baskayanak G, Peters UR, le Coutre P, Schmidt CA. Quantitative analysis of beta-actin, beta-2-n1icroglobulin and porphobilinogen deaminase mRNA and their comparison as control transcripts for RT-PCR. Mol Cell Probes 2002;16:25–30.
[13] Blanquicett C, Johnson MR, Heslin M, Diasio RB. Housekeeping gene variability in normal and carcinomatous colorectal and liver tissues: applications in pharmacogenomic gene expression studies. Anal Biochem 2002;303:209–14.
[14] Zhong H, Simons J. Direct comparison of GAPDH, β-actin, cyclophilin and 28SrRNA as internal standards for quantifying RNA levels under hypoxia. Biochem Biophys Res Commun 1999; 259:523–6.
[15] Aerts JL, Gonzales MI, Topalian SL. Selection of approriate control genes to assess expression of tumor antigens using real-time RT-PCR. Biotechniques 2004;36:84–6.
[16] Oikarinen A, Makela J, Vuorio T, Vuorio E. Comparison on collagen gene expression in the developing chick embryo tendon and heart. Tissue and development time-dependent action of dexamethasone. Biochim Biophys Acta 1991;1089:40–6.
[17] Tokunaga K, Nakamura Y, Sakata K, Fujimori K, Ohkubo M, Sawada K, et al. Enhanced expression of a glyceraldehydes-3-phosphate dehydrogenase gene in human lung cancers. Cancer Res 1987;47:5616–9.
[18] Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 2000;25:169–93.
[19] Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Meth 2000;46:69–81.
[20] Glare EM, Divjak M, Bailey MJ, Walters EH. Beta-actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels. Thorax 2002;9:765–70.
[21] Winer I, Jung CK, Shackel I, Williams PM. Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal Biochem 1999;270:41–9.
[22] Wall SJ, Edwards DR. Quantitative reverse transcription-polymerase chain reaction (RT-PCR): a comparison of primer-dropping, competitive, and real-time RT-PCRs. Anal Biochem 2002;300:269–73.
[23] Tatton WG, Chalmers-Redman RM, Elstner MW, Leesch W, Jagodzinski FB, Stupak DP, et al. Glyceraldehyde-3-phosphate dehydrogenase in neurodegeneration and apoptosis signaling. J Neural Transm Suppl 2000;60:77–100.
[24] Goidin D, Mammessier A, Staquet MJ, Schmitt D, Berthier-Vergnes O. Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and beta-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. Anal Biochem 2001;295: 17–21.
[25] Finnegan MC, Goepel JR, Hancock BW, Goyns MH. Investigation of the expression of housekeeping genes in non-Hodgkin's lymphoma. Leuk Lymphoma 1993;10:387–93.
[26] Solanas M, Moral R, Escrich E. Unsuitability of using ribosomal RNA as loading control for Northern blot analyses related to the imbalance between messenger and ribosomal RNA content in rat mammary tumors. Anal Biochem 2001;288:99–102.
[27] Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun 2004;313:856–62.
[28] Shen WC, Green MR. Yeast TAF(II)145 functions as a core promoter selectivity factor, not as a general coactivator. Cell 1997;90:615–24.
[29] Li B, Reese JC. Derepression of DNA damage-regulated genes requires yeast TAF(II)s. EMBO J 2000;19:4091–100.
[30] Acker J, Muroni O, Mattei MG, Kedinger C, Vigneron M. The gene (POLR2L) encoding the hRPB7.6 subunit of human RNA polymerase. Genomics 1996;32:86–90.
[31] Reichrath J, Welter C, Mitschele T, Classen U, Meineke V, Tilgen W, et al. Different expression patterns of calpain isozymes 1 and 2 (CAPN1 and 2) in squamous cell carcinomas (SCC) and basal cell carcinomas (BCC) of human skin. J Pathol 2003;199:509–16.
[32] Lee JH, Lee CW, Lee SG, Yang MS, Hong MK, Kim JJ, et al. Comparison of polymerase chain reaction with adenosine deaminase activity in pericardial fluid for the diagnosis of tuberculous pericarditis. Am J Med 2002;113:519–21.
[33] Gulec M, Akin H, Yuce H, Ergin E, Elyas H, Yalcin O, et al. Adenosine deaminase and xanthine oxidase activities in bladder washing fluid from patients with bladder cancer: a preliminary study. Clin Biochem 2003;36:193–6.
[34] Ozturk HS, Karaayvaz M, Kacmaz M, Kavutcu M, Akgul H, Durak I, et al. Activities of the enzymes participating in purine and free-radical metabolism in cancerous human colorectal tissues. Cancer Biochem Biophys 1998;l6:l57–168.
[35] Conolly GP. Hypoxanthine–guanine phosphoribosyltransferase-deficiency produces aberrant neurite outgrowth of rodent neuroblastoma used to model the neurological disorder Lesch Nyhan syndrome. Neurosci Lett 2001;(314):61–4.